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Macrophage functional diversity in NAFLD — more than inflammation

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Abstract | Macrophages have diverse phenotypes and functions due to differences in their origin, location and pathophysiological context. Although their main role in the liver has been described as immunoregulatory and detoxifying, changes in macrophage phenotypes, diversity, dynamics and function have been reported during obesity-related complications such as non-alcoholic fatty liver disease (NAFLD). NAFLD encompasses multiple disease states from hepatic steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocarcinoma. Obesity and insulin resistance are prominent risk factors for NASH, a disease with a high worldwide prevalence and no approved treatment. In this Review, we discuss the turnover and function of liver-resident macrophages (Kupffer cells) and monocyte-derived hepatic macrophages. We examine these populations in both steady state and during NAFLD, with an emphasis on NASH. The explosion in high-throughput gene expression analysis using single-cell RNA sequencing (scRNA-seq) within the last 5 years has revolutionized the study of macrophage heterogeneity, substantially increasing our understanding of the composition and diversity of tissue macrophages, including in the liver. Here, we highlight scRNA-seq findings from the last 5 years on the diversity of liver macrophages in homeostasis and metabolic disease, and reveal hepatic macrophage function beyond their classically described inflammatory role in the progression of NAFLD and NASH pathogenesis.

Macrophages are cells of the immune system most well-known for their ability to engulf big particles, to which they owe their name from the Greek *makrós phagein* (large eaters). They are present in essentially all organs of the body from embryonic to adult life[1]. Macrophages are highly plastic cells that sense, react and respond to their surrounding and systemic environment by changing their function, morphology and phenotype[2]. During immune activation, macrophages have a crucial role both in the innate, rapid, non-specific defence responses, and in the long-lasting and specific adaptive responses that involve the recruitment of other immune cells[2]. In addition to their phagocytic capacity, macrophages produce a plethora of factors such as cytokines, chemokines and growth factors, which are involved in the responses to infection and in tissue repair. In addition to their immunoregulatory roles in tissue homeostasis and defence against pathogens, macrophages limit the development of various diseases including sepsis, chronic inflammatory diseases (such as rheumatoid arthritis, colitis and psoriasis), neurodegenerative diseases and cancer[3]. In the past two decades, macrophages have also been extensively studied for their contribution to metabolic diseases, such as obesity-associated insulin resistance and diabetes mellitus.

The liver is an important metabolic organ in which macrophages reside and serve as a first line of defence against material from the gastrointestinal tract that passes through the liver via the portal circulation. While the liver is often described as the organ with the highest proportion of macrophages[4], this interpretation of the available literature is difficult to confirm as methods of macrophage extraction and subsequent analysis can greatly interfere with their recovery and identification. It is nevertheless clear that the liver contains a substantial number of macrophages, which contribute to both its maintenance and its function[4]. Changes in the dynamics of liver macrophage turnover and function have been described in non-alcoholic fatty liver disease (NAFLD)[5,6].

In this Review, we discuss the role of liver macrophages in steady state and in obesity-associated metabolic diseases, such as insulin resistance and NAFLD. We particularly concentrate on data generated using single-cell RNA sequencing (scRNA-seq) and focus on both the inflammatory and non-inflammatory roles of macrophages in the development of obesity-associated metabolic impairment in mice and humans. Finally, we use an integrated approach to present the similarities and discrepancies among scRNA-seq analyses aimed to

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Key points

- Macrophages are highly plastic cells of the immune system that can acquire a spectrum of phenotypes according to their spatiotemporal pathophysiological context.
- Liver macrophages are either embryo-derived resident macrophages or recruited peripheral monocyte-derived macrophages.
- Liver macrophages have been shown to contribute to non-alcoholic fatty liver disease (NAFLD) progression in obesity through production of both inflammatory and non-inflammatory factors.
- Single-cell RNA sequencing (scRNA-seq) has identified distinct liver macrophage subsets in mice and humans in health and liver disease.
- scRNA-seq has enabled the identification of novel pathogenic factors expressed by liver macrophages that could exacerbate or protect from NAFLD progression.

define liver macrophage populations and propose future directions to achieve harmony of studies in both mice and humans.

Physiological liver macrophage dynamics

Liver macrophages consist of tissue-resident Kupffer cells and recruited monocyte-derived macrophages from the systemic circulation[7,8]. Liver macrophages typically reside on the luminal side of the liver sinusoids, partly protruding into the perisinusoidal space where they are in close contact with hepatic stellate cells[9]. Liver macrophages are involved in tissue remodelling, responses to gut-derived pathogens, regulation of hepatocyte metabolism, iron and cholesterol metabolism and maintenance of immunological tolerance in the liver[7,10]. A 2017 study also identified a distinct subset of monocyte-derived macrophages (liver capsular macrophages) that reside in the hepatic capsule and function to protect the liver from pathogens derived from the peritoneum[11].

Turnover of mouse liver macrophages

Fate mapping studies in mice have been crucial in elucidating the turnover of liver macrophage subsets. These studies have shown that the majority (95%) of resident liver macrophages are Kupffer cells, which are derived from embryonic precursors from the yolk sac or the fetal liver[12,13]. Kupffer cells are seeded before birth and maintained by local proliferation, independently of the recruitment of bone marrow-derived circulating monocytes at steady state[4,14–16]. The proliferation of Kupffer cells occurs when there is a need for replenishment of liver macrophages. This process occurs through the transient downregulation of the transcription factors MafB and c-Maf, which repress macrophage-specific enhancers associated with a gene network that controls self-renewal[17].

Due to the large volume of blood that is continuously passing through the liver, the liver is highly accessible to monocytes from the systemic circulation that can enter and expand the pool of liver macrophages during conditions such as inflammation and metabolic disease or following Kupffer cell depletion[8,18]. However, these monocytes only differentiate into macrophages and gain tissue-specific functions when a niche is available[19]. In mice, circulating monocytes can be divided into two subsets: inflammatory monocytes and patrolling monocytes. The latter are monocytes that are recruited to non-inflamed tissues expressing Ly6C^{low} (CXCR1^{high}CCR2^{-}CD62L^{-}CD43^{high}) and mainly function to patrol the vasculature and healthy tissues.

Inflammatory monocytes are short-lived cells expressing Ly6C^{high} (CX_3CR1^{mid}CCR2^{+}CD62L^{+}CD43^{low}), and they respond to inflammatory signals such as monocyte chemoattractant protein 1 (MCP1, also known as CCL2), by migrating to the site of inflammation and differentiating into monocyte-derived macrophages and monocyte-derived dendritic cells[20–22]. Upon recruitment to the liver, inflammatory monocytes can acquire a similar phenotype to resident Kupffer cells when an empty niche is available[8,23].

The local tissue environment is important in shaping the phenotype of recruited macrophages, as specific signals from the liver niche drive the development and maintenance of resident monocyte-derived macrophages during homeostasis[9,24]. However, not all infiltrating monocytes differentiate into long-lived resident macrophages, and, in some instances (for example, during inflammation or loss of resident macrophages), recruited monocytes differentiate into short-lived macrophages that only temporarily reside in the liver[9,25,26]. Moreover, mature peritoneal cavity macrophages (expressing F4/80^{+}GATA6^{+}) have previously been shown to infiltrate the liver upon sterile injury, where they then acquire a restorative phenotype[27]. However, this finding is currently debated as another study demonstrated that, although peritoneal macrophages accumulate on the liver surface in response to injury, they do not invade the liver tissue[28]. Taken together, these studies have, however, demonstrated that recruited liver macrophages can arise from different sources and that their fate depends on their origin and localization (FIG. 1).

Turnover of human liver macrophages

While the ontogeny and replenishment of liver macrophages have been extensively studied in mouse models[12,16,17], little is known about the origin and replenishment of their human counterparts. In humans, circulating monocytes are classified by expression of either CD14^{+}CD16^{low} (classic monocytes), CD14^{+}CD16^{+} (intermediate monocytes) or CD14^{low}CD16^{+} (non-classic monocytes)[29,30]. Classic monocytes are short-lived cells (1–2 days) that make up 85% of all circulating monocytes, and are thought to be the main source of both CD14^{+}CD16^{+} intermediate monocytes and monocyte-derived macrophages in tissues[31,32]. Human classic monocytes, corresponding to inflammatory Ly6C^{high} monocytes in mice, are maintained in reserves in the bone marrow and spleen at steady state, where they are readily available upon infection or injury. Non-classic monocytes, the counterpart to patrolling Ly6C^{low} monocytes in mice, reside for longer in the circulation where they patrol and are involved in maintaining tissue homeostasis. Intermediate monocytes do not patrol the vasculature; however, they share characteristics of both classic and non-classic monocytes[31]. Although studies have shown that human monocytes can infiltrate into tissues during inflammation[33–35], similar to monocytes in mice, the fate of these cells after the resolution of inflammation is unclear. Additionally, it is not currently known

Fate mapping
Labelling of specific cell subsets in the embryo to trace their contribution to cell populations and tissues in the adult organism.

Yolk sac
A sac attached to the embryo during development that provides nutrients and cells to the embryo.

Sterile injury
Injury or inflammation not caused by pathogenic infection.

if infiltrating monocytes can differentiate into resident macrophages in the human liver.

Despite the lack of tools to examine the turnover of tissue macrophages in humans, there is evidence supporting the role of resident monocyte-derived macrophages in human liver. A few studies have examined monocyte recruitment to the liver in patients undergoing liver transplantation. Using immunohistochemical staining of $CD68^+$ macrophages in liver tissues from men who received liver transplants from donors who were women, a small proportion (16% of total liver macrophages) of donor-derived (XX^+) resident macrophages remained for years after transplantation. It was proposed that these resident cells were maintained by self-renewal[36]. Interestingly, the same study demonstrated that the majority (80%) of $CD68^+$ liver macrophages had been replaced by macrophages derived from circulating monocytes from the recipient (Y^+) more than 1 year after transplantation. This finding indicates that newly recruited monocytes also have the ability to differentiate into resident macrophages and reside in the human liver for years.

Another study examined the replacement of donor-derived liver macrophages by monocyte-derived cells from the recipient after liver transplantation, whereby donor-specific and recipient-specific macrophages were differentiated by their HLA type. This study revealed the presence of a subpopulation of long-lived resident macrophages characterized by a distinct surface marker signature ($CD68^{high}CD206^{high}CD163^{high}CX_3CR1^{high}$). Although the study did not show any data relating to the quantity of these resident macrophages, a proportion of this macrophage population remained of donor origin for up to 10 years following liver transplantation. This finding suggests that these long-lived macrophages could be capable of self-maintenance independent of renewal from circulating monocytes[37].

Taken together, these studies highlight that both humans and mice possess a mixed pool of both resident and recruited macrophages. Unlike in mice, however, the majority of human liver macrophages are derived from circulating monocytes during steady state (FIG. 1). Whether these differences in resident and recruited macrophage proportions are species-specific or are caused by environmental differences between mice and humans remains to be determined. For example, in the aforementioned studies using mice, the animals (as are laboratory animals generally) were genetically homogeneous, lived in protected facilities and had limited contact with pathogens. In contrast, humans are constantly exposed to new pathogens and other inconsistencies in the environment, including diverse diets, and it is plausible that this exposure would elicit a higher turnover of tissue macrophages. It would be interesting to utilize wild mice or similar models to test this hypothesis.

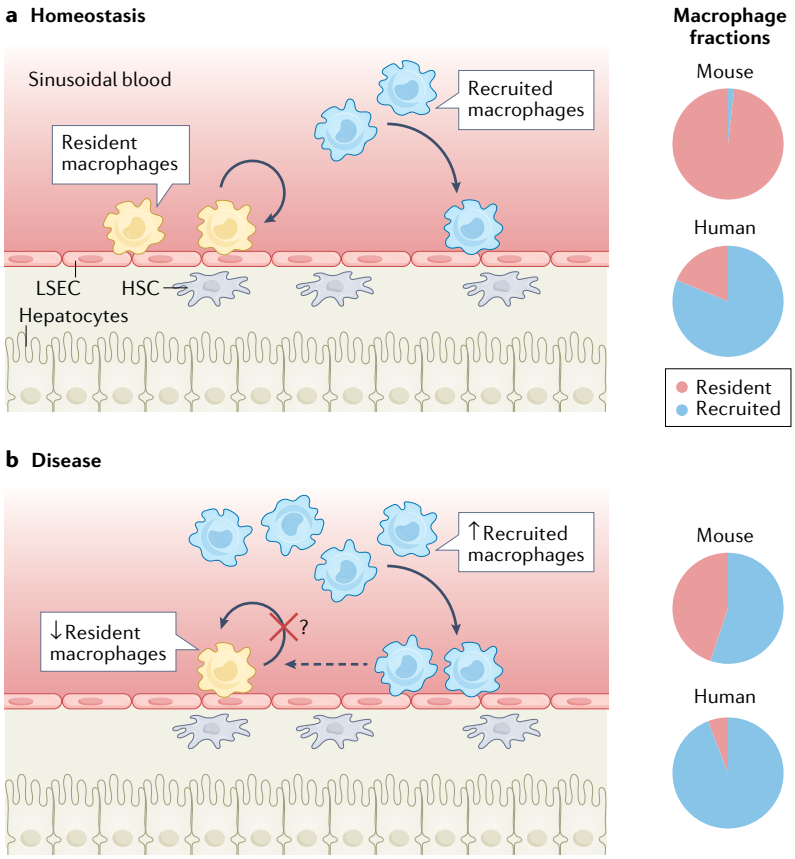
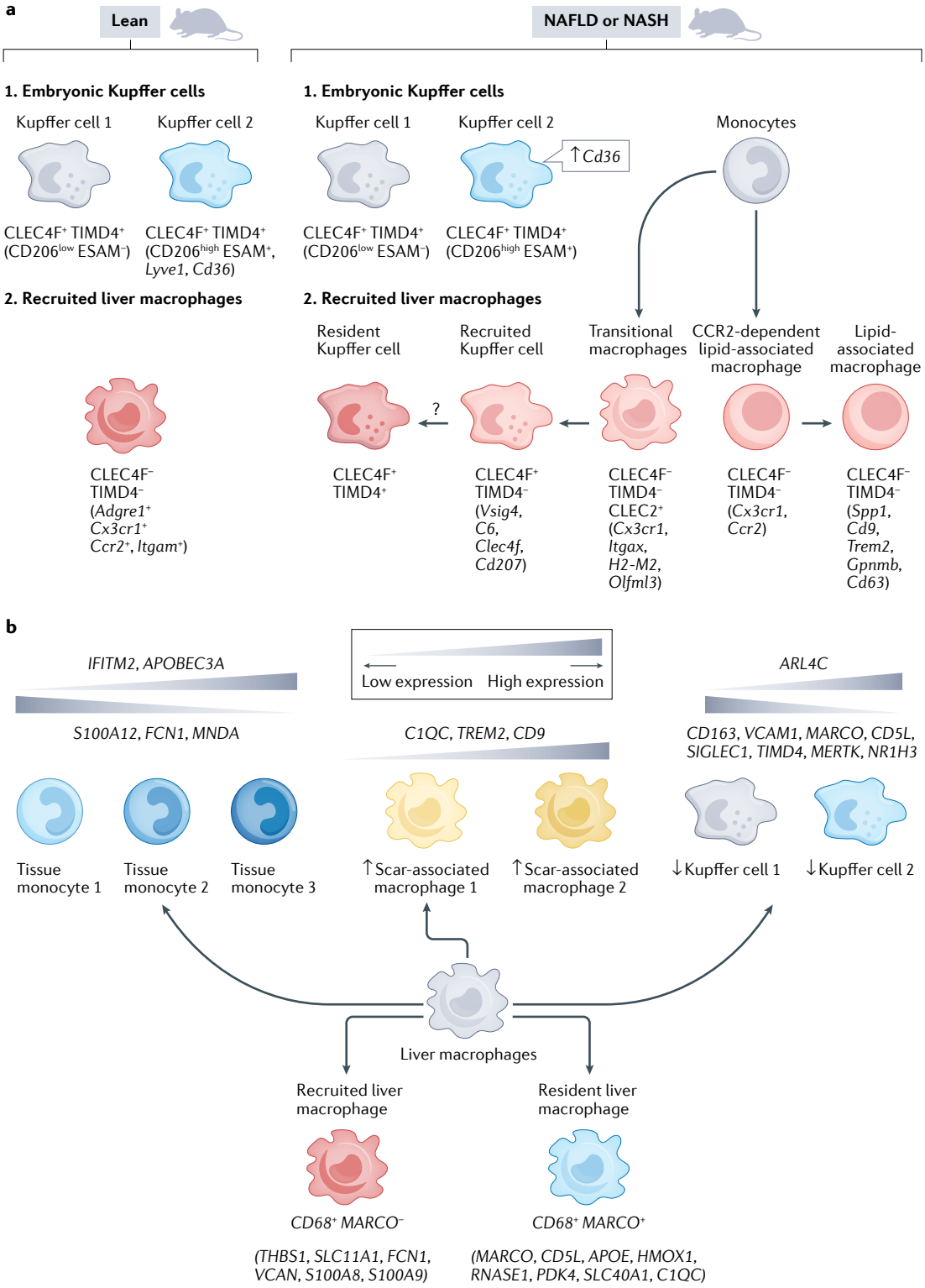


Fig. 1 | **Liver macrophage turnover in health and disease. a** | During homeostasis, the majority of mouse liver macrophages consist of embryo-derived resident macrophages, also known as Kupffer cells. These Kupffer cells are localized in the liver sinusoids and maintained locally through proliferation. Mouse livers also contain a small proportion of recruited monocyte-derived macrophages, found to partly localize near large vessels. By contrast, human livers consist of both resident and recruited monocyte-derived macrophages at steady state, with the proportion of recruited macrophages being greater than in mice. Moreover, these recruited macrophages have been suggested to localize close to the central vein in the healthy liver, while resident macrophages localize at periportal regions. **b** | Little is known about liver macrophage turnover during the early stages of non-alcoholic fatty liver disease (NAFLD) and hepatic steatosis. During non-alcoholic steatohepatitis and cirrhosis, resident liver macrophages decrease in number in both humans and mice. This reduction has been associated with dysfunctional self-renewal in mice, although the exact molecular mechanism of how self-renewal of resident macrophages is affected by NAFLD remains to be elucidated (indicated by the question mark). To compensate for the loss of resident macrophages the recruitment and infiltration of monocytes to the liver is increased, leading to more recruited monocyte-derived macrophages. In mice, these newly recruited macrophages can acquire a similar phenotype to resident liver macrophages (indicated by the dashed arrow) or remain as monocyte-derived macrophages. Moreover, this reduction in resident liver macrophage numbers has not been observed during earlier stages of steatosis, indicating that these changes are specific to the development of fibrosis. HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell.

Diversities of hepatic macrophages

scRNA sequencing of human liver macrophages. Rapidly evolving single-cell transcriptomic and proteomic technologies have revolutionized high-resolution analyses of cellular composition and heterogeneous cell states and have substantially advanced our understanding of the pathogenesis of several diseases. Various scRNA-seq methods have been specifically developed to understand cellular heterogeneity and have been used in studies of the liver (Supplementary Table 1). These methods differ in how the single cells are isolated and converted into cDNA libraries for sequencing. While plate-based methods such as Smart-seq2 and Smart-seq3 (REFS[38,39]) and Cel-Seq2 (REF.[40]) have the major advantage of a higher gene detection rate than droplet-based methods[41], most liver scRNA-seq studies have



used droplet-based approaches, such as 10x Genomics Chromium, to achieve high-throughput cellular yields[42] (Supplementary Table 2). These unbiased studies have allowed the identification of most cell types present in the liver and even subpopulations within the same cell type[43], which had not been previously possible using traditional methods such as flow cytometry or immunofluorescence.

A 2018 scRNA-seq study of healthy human livers by MacParland et al. revealed distinct liver macrophage populations, which are stratified into two major subsets of *CD68*$^{+}$*MARCO*$^{+}$ and *CD68*$^{+}$*MARCO*$^{-}$ cells[44] (FIG. 2). The *CD68*$^{+}$*MARCO*$^{+}$ macrophages are immunoregulatory cells, expressing *VCAM1*, *CD5L*, *HMOX1*, *MRC1*, *CD163*, *M4SA7* and *VSIG4*, similar to the long-lived resident Kupffer cells identified in mice[8,44]. In humans,

◀ Fig. 2 | **scRNA-seq studies in mouse and human livers revealed distinct macrophage subsets.** scRNA-seq revealed macrophage subpopulations within the liver during health and disease. **a** | In mice, three distinct populations of macrophages have been identified in healthy liver. The majority of these are resident, embryo-derived, macrophages, which have been characterized into a major population of CD206^{low}ESAM^{-} Kupffer cell 1 and a minor population of CD206^{high}ESAM^{+} Kupffer cell 2 (REF.[55]). Mouse liver also contains a minor population of CLEC4F^{-}TIM4^{-} recruited liver macrophages in steady state[13,51,52,54]. In non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) the population of recruited macrophages is expanded and can be divided into either lipid-associated macrophages or CCR2-dependent lipid-associated macrophages with a lipid handling phenotype, or into CLEC2^{+} transitional macrophages. CLEC2^{+} transitional macrophages have been shown to develop into monocyte-derived macrophages with a phenotype similar to that of resident, embryo-derived, TIM4^{+} Kupffer cells[8,13,51,52,54,57]. However, in NASH, the majority of recruited Kupffer cells have been found to remain partly immature and do not appear to develop into fully functional and mature resident Kupffer cells (indicated by the question mark), although why this is remains unclear. **b** | In humans, two major populations of macrophages have been identified in healthy liver[44,46,48,49]. These consist of either recruited *CD68*$^{+}$*MARCO*$^{-}$ or resident *CD68*$^{+}$*MARCO*$^{+}$ liver macrophages. Another study characterized these populations into even more subsets, with two populations of resident *CD68*$^{+}$*MARCO*$^{+}$ macrophages (called Kupffer cell 1 and Kupffer cell 2) with gradual differences in their expression of resident macrophage marker genes[45]. The same study also characterized two subsets of recruited liver macrophages (called scar-associated macrophages 1 and scar-associated macrophages 2) and three populations of tissue monocytes in healthy livers with gradual differences in marker genes[45]. During liver cirrhosis, both populations of recruited scar-associated macrophages increase in proportion, while resident Kupffer cell populations decrease in the liver. The origin and markers are listed for each subpopulation.

a subpopulation of proliferating *CD68*$^{+}$*MARCO*$^{+}$ Kupffer cells have been identified, indicating that human Kupffer cells undergo self-renewal[45]. The *CD68*$^{+}$*MARCO*$^{-}$ subset comprise recruited liver macrophages and they express high levels of *S100A8*, *LYZ*, *S100A9*, *S100A12*, *VCAN* and *S100A6*, a signature that suggests they are monocyte-derived[44]. While *CD68*$^{+}$*MARCO*$^{-}$ cells were described as being more pro-inflammatory than *CD68*$^{+}$*MARCO*$^{+}$ resident cells, these cells seemed to be less inflammatory than circulating *CD16*$^{+}$ monocytes[46]. This finding suggests that, upon entry into the liver, monocytes adapt to an immunotolerant environment that limits their inflammatory activation. However, this study compared the transcriptomic profiles of tissue-resident macrophages from liver and spleen with that of circulating monocytes[46]. A comparison of only liver macrophages and peripheral monocyte populations is therefore warranted.

A 2019 scRNA-seq study of healthy and cirrhotic livers by Ramachandran et al. characterized human liver macrophages into seven distinct populations[45], including three subsets of tissue monocytes (tissue monocyte subsets 1–3), two scar-associated macrophages subsets (scar-associated macrophage subsets 1 and 2) and two subsets of resident liver macrophages (Kupffer cell subsets 1 and 2), as presented in FIG. 2. While the proportions of *TREM2*$^{+}$ and *CD9*$^{+}$ scar-associated macrophages characterized in this study were statistically significantly higher in the fibrotic liver, the resident liver macrophages were more abundant in the healthy livers. The Kupffer cell subsets 1 and 2 were both *CD68*$^{+}$ and *MARCO*$^{+}$, similar to the resident liver macrophages characterized in the study by MacParland et al.[44]. The two Kupffer cell subsets were distinguished from each other by gradual differences in their expression levels of macrophage signature genes as Kupffer cell 1 cells expressed higher levels of resident macrophage genes than Kupffer cell 2 cells (REF.[45]). While other studies have also revealed two populations of resident liver macrophages[47–49], these populations seem to mostly differ based on their origin. In addition, integration analyses would be required to clearly define the similarities or the lack thereof between these studies.

Although these studies have greatly advanced our knowledge of macrophage turnover and diversity in the human liver, many challenges remain. The first is that there is substantial variability in the number of different subpopulations of macrophages, even between healthy individuals, which could affect the distribution and transcriptomic analyses of these populations. The second is the number of individuals sampled in each study. Large-scale scRNA-seq studies are inherently challenging to perform due to the limited accessibility of human liver samples and the cost associated with these studies. The third challenge relates to what the definition of 'healthy' or 'control' group means in liver scRNA-seq studies. It is common practice to use livers from deceased individuals, for whom there is limited clinical information regarding hospitalization and medication prior to tissue collection. In addition, these livers are usually livers that have been rejected for organ transplantation, making it difficult to delineate whether these samples truly reflect the healthy state. Finally, the size of the biopsy used might limit the possibility of removing the intrahepatic blood, and so circulating monocytes might contaminate the tissue monocyte fraction. While these are sizeable caveats to consider, additional human studies are essential in order to improve the translatability of research in mice and to facilitate better understanding of liver macrophage biology in health and disease.

Mouse liver macrophage diversity. scRNA-seq studies have also revealed distinct macrophage populations in the mouse liver, the majority of which are Kupffer cells[13,50–54] (FIG. 2a). These Kupffer cells express high levels of the tissue macrophage markers *Adgre1* (encoding F4/80), *Clec4f* and *Timd4*, as well as a low level of *Itgam* (encoding CD11b). Under physiological conditions F4/80^{high}CD11b^{low}Tim4^{+} embryo-derived resident liver macrophages expressing *Clec4f* at the mRNA and the protein levels are predominant[8,14]. Moreover, embryo-derived Kupffer cells have further been divided into two functionally distinct subsets, including CD206^{low}ESAM^{-} Kupffer cell population 1 and a minor population of CD206^{+}ESAM^{+} Kupffer cell 2 cells[55,56] (FIG. 2a). Conversely, recruited macrophages are less numerous in healthy livers and are derived from circulating bone marrow-derived monocytes[13,52]. These recruited macrophages can be divided into two main populations of either CLEC4F^{+}TIM4^{-}CLEC2^{+} monocyte-derived Kupffer cells or CLEC4F^{+}TIM4^{-}CLEC2^{-} monocyte-derived macrophages[52,57]. As human liver-resident *TIMD4*$^{+}$ and recruited *TIMD4*$^{-}$ liver macrophages are present in similar proportions under healthy conditions, the disparity in mouse proportions further suggests a lower turnover of tissue macrophages in the mouse liver

Tissue monocytes
Cell population identified in the liver tissue by single-cell RNA sequencing (scRNA-seq) with a phenotype similar to circulating monocytes.

Scar-associated macrophages
Liver macrophages with a lipid-handling phenotype mainly associated with the development of liver fibrosis in patients with cirrhosis.

Tissue-specific enhancer
A regulatory element that induces tissue-specific transcription factors shaping the phenotype of tissue-resident cells.

Lipid-associated macrophages
Tissue macrophages with a lipid handling phenotype mainly associated with the development of obesity and non-alcoholic steatohepatitis.

Crown-like structures
Histological finding where macrophages are surrounding lipid-laden hepatocytes (in the liver) or dying adipocytes (in the adipose tissue) in a crown-like structure.

compared than in the human liver[44,49]. It is worth noting that, while *TIMD4* expression has been previously used as a marker of resident liver macrophages in both humans and mice, differential expression of *TIMD4* was found to distinguish populations of resident liver macrophages in a different study[45].

Liver macrophage cellular interactions

Macrophages exhibit distinct transcriptional programmes defined by tissue-specific enhancer landscapes[58]. The niche tissue environment is thus important for their tissue-specific identity. The Kupffer cell-specific identity is shaped by the co-operative efforts of liver sinusoidal endothelial cells, hepatic stellate cells and hepatocytes. These cells express the ligands CSF1, DDL4, TGFβ, BMP9 and desmosterol, which activate transcription factors such as LXRα, SPIC, RBPJ, SMADs and ID3 in liver macrophages, leading to the initiation and maintenance of Kupffer cell-specific transcriptional programmes[9,24,53]. Another factor found to be expressed by hepatic stellate cells at the Kupffer cell niche is IL-34, but its role in the liver during steady state has not yet been functionally validated[9]. The cooperation of transcription factors in regulating the expression of target genes requires open chromatin accessibility, which is mediated via epigenetic mechanisms. Elucidating the diversity in the epigenome could thus reveal the underlying mechanisms that drive the regulation of macrophage phenotypes in health and metabolic disease.

Liver macrophage zonation

Monocytes and macrophages are well known for their intrinsic plasticity, which allows them to rapidly modify their morphology in response to their microenvironment[2]. Due to their ability to migrate, a single monocyte can potentially integrate and differentially respond to different surrounding cells in various locations within the same tissue. A 2004 study using immunohistochemistry and digitonin–collagenase perfusion isolation suggested that Kupffer cells exhibit functional differences in the periportal and perivenous zones[59]. A 2021 study using quantitative multiplex confocal imaging and mathematical modelling to assess the localization of immune cells in the liver revealed that Kupffer cells concentrate around periportal regions[60]. Immune zonation was observed to be a dynamic process dependent on the gut microbiota, and liver sinusoidal endothelial cells were determined to be key cells in the immune zonation process by influencing chemokine gradients[60]. In humans, MARCO$^+$ cells were found in increased numbers in the periportal regions compared with other areas of the healthy liver[44]. This finding was further confirmed by spatial transcriptomics showing that human *CD68*$^+$*MARCO*$^+$ resident liver macrophages localized to periportal regions while recruited *CD68*$^+$*MARCO*$^-$ liver macrophages tended to be closer to central veins[49]. Mouse studies, however, have failed to reveal any particular zonation of macrophages other than the observation that some monocyte-derived macrophages are located at the large vessels, probably due to their infiltration path[9,24,52,55]. In the context of non-alcoholic steatohepatitis (NASH), lipid-associated macrophages have been found to aggregate into crown-like structures or in fibrotic regions[52,61]. The study of macrophage zonation could greatly benefit from multiplex in situ analyses, which would combine the detection of multiple markers of each subpopulation at both the mRNA and protein levels.

Liver macrophages in NAFLD and NASH

Macrophages have been extensively studied in the context of both acute inflammatory conditions (such as sepsis and wound healing) and chronic inflammatory disorders (such as rheumatoid arthritis and colitis)[3]. We now discuss the diverse roles of liver macrophages in the regulation of systemic metabolism during the development of metabolic disease.

Obesity and NAFLD

NAFLD is characterized by the excessive accumulation of lipids (triglycerides) in the liver. These lipids can arise from diet, can be produced de novo by the liver itself, or can arise from adipose tissue that releases lipids into the bloodstream[62]. NAFLD has now become the most common liver disease in westernized countries[63]. Although NAFLD is highly associated with obesity (approximately 50% of patients with NAFLD have obesity) and obesity-related metabolic conditions including type 2 diabetes mellitus (about 20% of patients with NAFLD) and dyslipidaemia (about 70% of patients with NAFLD), lean individuals can also develop NAFLD[64,65].

NAFLD represents a spectrum of liver conditions with the possible outcomes of liver failure and hepatocellular carcinoma[66]. Abnormal lipid accumulation in the liver, also called hepatic steatosis, can further advance into NASH, which is associated with cellular stress and hepatocyte injury. This cellular injury can lead to cell death, inflammation and fibrosis[67,68]. Patients with NAFLD and NASH can present a variable combination of all of these different conditions, and NAFLD is thus considered a multifactorial disease with highly heterogeneous clinical manifestations[69,70]. Moreover, insulin resistance has been suggested to be an independent risk factor for the progression of NAFLD, since insulin resistance in both the adipose tissue and the liver leads to an accumulation of free fatty acids in hepatocytes[71,72]. This accumulation is thought to further increase the occurrence and toxicity of additional stresses to the liver, such as oxidative stress, endoplasmic reticulum stress, dysfunctional unfolded protein responses and induction of inflammation. These stresses result in hepatocyte injury gradually accumulating over time[73–75].

Role of liver macrophages in NAFLD

Inflammatory role of liver macrophages in NAFLD. The importance of liver macrophages in metabolic disease was first suggested using mice and rats that were depleted of liver macrophages using either clodronate liposomes or gadolinium chloride. This depletion of liver macrophages prevented the development of hepatic steatosis and insulin resistance in obese mice and rats[76,77]. The beneficial effect of liver macrophage depletion on hepatic insulin sensitivity was initially attributed to a decrease in the hepatic levels of inflammatory cytokines[78]. However, most

Liver non-parenchymal cells
Liver cells that do not make up the liver parenchyma including liver sinusoidal endothelial cells, hepatic stellate cells and immune cells.

studies investigating liver macrophage activation in the context of obesity and insulin resistance were based on whole liver[79,80] or inflammatory gene expression in all liver non-parenchymal cells[81], depletion approaches[76,82–86] or myeloid-specific inflammatory gene ablation[87,88]. These experimental approaches could lead to the extrapolation of observations from adipose tissue macrophages rather than liver macrophages alone. Gene expression analyses have been helpful in describing the inflammatory phenotype of liver macrophages. RNA sequencing of recruited and resident liver macrophages in mice fed a high-fat diet for 15–20 weeks has shown that inflammatory markers are only expressed in monocyte-derived liver macrophages during obesity and hepatic steatosis, not in resident Kupffer cells[89]. However, these two populations were only defined according to their levels of expression of the surface markers F4/80 and CD11b by flow cytometry (recruited F4/80^{low}CD11b^{+} compared with resident F4/80^{high}CD11b^{+}), which does not allow discrimination between different subsets of resident and recruited liver macrophages.

Additional transcriptomic profiling of liver macrophages based on their origin (embryo-derived versus monocyte-derived) confirmed a higher inflammatory status of recruited, monocyte-derived, macrophages in mice fed a NASH-inducing methionine–choline-deficient diet for 6 weeks compared with embryo-derived macrophages[57]. In both macrophage populations, genes involved in lipid metabolism were among the most regulated in NASH. Interestingly, this study highlighted the infiltration of monocytes following a decrease in resident macrophage content that was occurring in NASH, but not in simple steatosis. This finding raised the question as to whether monocyte infiltration and therefore inflammation only occurs during severe fatty liver disease stages such as NASH. In accordance with this hypothesis, our analyses of the transcriptomic profile of liver macrophages in patients with obesity with simple steatosis failed to reveal any inflammatory activation of these cells[90]. However, these studies did not fully capture the diversity of liver macrophages in health and disease. Recently, the utilization of next generation sequencing at the single-cell level has allowed the in-depth study of macrophage subpopulation phenotypes and their contribution to metabolic impairments associated with obesity[91]. These scRNA-seq approaches have allowed the distinction of cells according to their origin, function and associated inflammatory phenotype.

Overall, the liver inflammation mediated by macrophages seems to also be highly dependent on the recruitment of monocytes and the severity of the liver disease. Supplementary Table 1 lists all the scRNA-seq studies of liver macrophages in health or liver disease with associated durations and types of diets. These scRNA-seq studies analysed the transcriptomic profile of liver macrophage subpopulations in mouse models of diet-induced NASH. NASH was induced by feeding mice with high levels of fat (40–60%), sugar (20–22% fructose or 10% sucrose in the diet or 13 g/l of fructose in the drinking water) and, in some cases, cholesterol (1–2%) for at least 16 weeks. Monocyte-derived liver macrophages were determined to be inflammatory, while embryo-derived macrophages either did not harbour an inflammatory phenotype or were slightly more inflammatory than their healthy controls[13,51,52,54,61]. These studies therefore confirmed the association between inflammation and monocyte recruitment following long-term feeding with a diet inducing severe stages of NAFLD or NASH[89,90].

Role of liver macrophages in NAFLD beyond inflammation. The first indication that liver macrophages could contribute to the metabolic impairment induced by obesity independently of inflammatory cytokines is that insulin resistance and steatosis occur before the onset of inflammation in the liver of obese mice[90] (FIG. 3). In addition, depletion of liver macrophages only results in a beneficial effect on insulin sensitivity when performed at an early time point of high-fat feeding and thus in the absence of inflammatory activation or recruitment of pro-inflammatory macrophages[76,77] (FIG. 3). Interestingly, specific silencing of the master regulator of inflammation, *Nfkb*, in liver macrophages reduced IL-1β expression and improved hepatic insulin sensitivity in obese mice, suggesting a direct role of liver macrophage-derived IL-1β in obesity-induced liver dysfunction[92]. However, specifically silencing *Il1b* in liver macrophages using the same methodology failed to improve hepatic insulin sensitivity in obese mice[90]. This finding suggests that NF-κB in liver macrophages could regulate hepatic insulin sensitivity in obese mice independently of IL-1β. It would thus be interesting to study the downstream transcriptional regulation of NF-κB upon silencing in liver macrophages, and to validate the function of its target genes in insulin sensitivity.

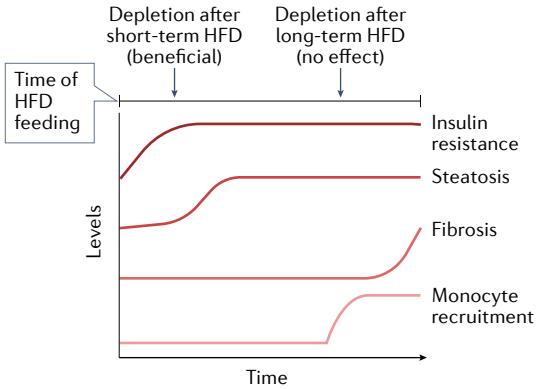


Fig. 3 | **Liver macrophage depletion and obesity-associated effects in mice.** Disease progression and monocyte recruitment during high-fat diet (HFD) feeding in mice. Liver macrophage depletion during short-term (0–4 weeks), but not long-term (14–16 weeks), high-fat diet feeding is beneficial in obesity, resulting in a reduction in insulin resistance and steatosis, and prevention of monocyte recruitment and fibrosis. This finding highlights an important role of liver macrophages in the emergence of insulin resistance but a minor role in its maintenance. In addition, monocyte recruitment, thought to be inflammatory, occurs after the development of insulin resistance and steatosis. It remains to be tested whether monocyte recruitment is a key component of the development of fibrosis.

Contrary to the prevailing dogma, our report from 2019 demonstrated that liver macrophages do not become pro-inflammatory during obesity and insulin resistance in mice and humans[90]. As mentioned earlier in this Review, the duration of the diet that induces NAFLD is of crucial importance for the inflammatory phenotype of liver macrophages. Indeed, in this study[90] mice fed a high-fat diet developed obesity, hepatic steatosis and insulin resistance after 9 weeks, but inflammatory monocytes were not recruited until at least 12 weeks of high-fat diet feeding. However, liver macrophages were suggested to contribute to the development of metabolic disease by producing the factor insulin-like growth factor-binding protein 7 (IGFBP7), which directly regulates insulin signalling and lipid accumulation in obese mice and humans with obesity[90]. Although liver macrophages were not activated after high-fat diet feeding, they did acquire a different phenotype to liver macrophages from healthy controls, with more than 1,000 genes statistically significantly regulated by obesity-induced insulin resistance[90]. In both mice and humans, most of the genes regulated in liver macrophages upon obesity are involved in oxidative stress[93]. This observation is not surprising since obesity is often associated with the ectopic deposition of lipids in the liver, leading to peroxidation and oxidative stress. In the same study, liver macrophages were shown to contribute to oxidative stress in obese livers as a result of dysfunctional lipid peroxidation and excessive production of reactive oxygen species[93].

Immune cells can also have beneficial effects on insulin sensitivity[83,87,94,95]. A few studies have identified liver macrophages with anti-inflammatory phenotypes maintained by the fatty acid sensor peroxisome proliferator-activated receptor-δ (PPARδ)[87]. Moreover, these anti-inflammatory liver macrophages induced apoptosis of pro-inflammatory liver macrophages via caspase 3, thereby preventing their pathological effects during NAFLD[96]. Liver macrophages also play beneficial roles during the development of liver fibrosis, whereby pro-fibrolytic Ly6C^{low} monocyte-derived macrophages induce apoptosis of activated hepatic stellate cells, along with secretion of matrix metalloproteinases (such as MMP9 and MMP12) and IGF1 that degrade the extracellular matrix[97,98].

Box 1 | **Therapeutic approaches targeting liver macrophages**

- **Galectin 3 inhibitors:** Galectin 3 is mainly expressed by macrophages and its inhibition has beneficial effects on fibrosis development. The inhibitor belapectin (GR-MD-02) was tested in clinical trials and was effective in reducing portal hypertension and fibrosis[123]. Another trial is currently ongoing to confirm these results[123].
- **C-C chemokine receptor 2 (CCR2) and CCR5 inhibitors:** CCR2 and CCR5 inhibitors such as cenicriviroc block CCR2-dependent and CCR5-dependent infiltration of monocytes to the liver. Clinical trials demonstrated that cenicriviroc has positive effects on liver fibrosis but is not effective at preventing the progression of hepatic steatosis. The phase III trial was therefore terminated for lack of efficacy[103].
- **Peroxisome proliferator-activated receptor (PPAR) ligands:** PPARs are fatty acid sensors that regulate many of the pathways implicated in NAFLD, NASH and metabolic disease. Activation of PPARβ and/or PPARδ inhibits activation of liver macrophages and maintains their anti-inflammatory status. Previous attempts to target individual PPARs have shown limited effect, and ongoing clinical trials are instead using either dual PPAR agonists (such as elafibranor) or pan-PPAR agonists (such as lanifibranor) to broadly target PPAR-related pathways associated with NAFLD and/or NASH[104,105].

Liver macrophage turnover in NAFLD

Although the number of liver macrophages does not appear to change during early stages of obesity and insulin resistance in mouse models[57,90], there are some indications that obesity-induced insulin resistance is associated with dysfunctional self-renewal of resident liver macrophages. For instance, increased proliferation of liver macrophages has been associated with the development of insulin resistance and oxidative stress[99]. This finding is further corroborated by the fact that, in mouse models of more severe liver disease such as NASH, Kupffer cells have been suggested to undergo cell death due to impaired self-renewal[57]. In mice this cell death has been associated with reduced proliferation of resident liver macrophages and increased recruitment of Ly6C^{+} inflammatory monocytes. These inflammatory monocytes develop into monocyte-derived macrophages in order to maintain the Kupffer cell pool during chronic liver disease[13,52,57]. However, these newly recruited monocyte-derived macrophages appear to be more immature and more inflammatory with an altered metabolic phenotype compared with bona fide Kupffer cells, promoting both hepatocyte damage and fibrosis during disease[100–102].

Monocyte-derived resident liver macrophages have also been suggested to acquire a distinct pathogenic lipid-associated phenotype in human livers during cirrhosis[45]. The chemokine receptor inhibitor cenicriviroc was designed to target the recruitment of monocyte-derived macrophages as an anti-NASH therapeutic. However, cenicriviroc failed in clinical trials due to a lack of efficacy[103]. Although the details of the study are not yet disclosed, it is worth noting that this finding suggests that blocking monocyte recruitment using antagonists of chemokine receptors is not a promising therapeutic approach for NASH and liver fibrosis. Other attempts to target liver macrophages to treat NASH, whether directly or indirectly, have also been explored (BOX 1), including PPAR activators to block the inflammatory activation of liver macrophages. While most clinical trials have demonstrated limited efficacy of these drugs, the pan-PPAR agonist lanifibranor (an agonist of PPARα, PPARγ and PPARδ) has demonstrated promising results, supporting further assessment in a phase III trial[104,105]. Macrophages have so far been solely explored as therapeutic targets for their ability to produce inflammatory cytokines. However, a growing body of evidence suggests that they might have a role in regulating liver metabolism or fibrosis independently of their inflammatory status. Additionally, there are discrepancies between data from preclinical models (particularly in mice) and human data, such as differences in macrophage functional diversity. scRNA-seq approaches in human livers might therefore improve our understanding of macrophage diversity in liver diseases and pave the way to better therapeutic approaches targeting particular functions in distinct macrophage populations for NASH and fibrosis.

Overall, these data suggest that loss of resident liver macrophages might also occur during early stages of liver disease, including obesity-induced insulin resistance. In this situation, a reduction of resident macrophages in response to obesity-induced metabolic stresses would be

Temporal trajectory analysis
In silico analysis of cell trajectories or cell differentiation patterns based on scRNA-seq data.

compensated by increased proliferation of the remaining resident macrophages. However, it remains to be elucidated why the monocyte-derived macrophages that infiltrate the liver during later stages of NAFLD respond differently to metabolic disease compared with their embryonic counterparts. It is also necessary to determine whether or not the pathogenic phenotype seen in these monocyte-derived macrophages is acquired upon entry to the liver in response to the pathogenic liver niche, or if monocytes are already programmed with this phenotype as bone marrow precursors, as has been previously suggested[51]. Finally, as the majority of these studies were conducted using mouse models that do not fully recapitulate disease development in humans, it is difficult to draw conclusions about the role of different macrophage subsets in the regulation of obesity-induced metabolic disease in humans.

Liver macrophage populations in NAFLD

scRNA sequencing of liver macrophages in NAFLD. As described earlier in this Review, macrophages display distinct phenotypes in response to signals from their environment, which include metabolic and immune fluctuations occurring during liver disease. scRNA-seq studies have revealed multiple populations of macrophages within the liver during health and metabolic diseases. The different markers of these populations are summarized in FIG. 2. Studies analysing liver macrophage diversity at the single-cell level in metabolic diseases have focused on the late stages of NAFLD. In particular, these studies have focused on NASH in mice and liver cirrhosis in humans. Since there are few scRNA-seq studies of early stages of NAFLD, we discuss the dynamics of liver macrophage diversity in the context of the later disease stages.

In mouse models of NASH, the proportion of monocyte-derived macrophages relative to the total number of liver macrophages increases dramatically throughout NAFLD progression (from approximately 10% in the healthy liver to 50% during NASH). This increase in monocyte-derived macrophages shifts the balance of resident macrophages versus recruited macrophages in favour of recruited macrophages[13,52,57,61,106]. These recruited macrophages comprise transitional macrophages, monocyte-derived Kupffer cells and lipid-associated macrophages, including CCR2-dependent lipid-associated macrophages[13,52,61]. While the lipid-associated macrophages exhibit impaired functions and an increased pro-inflammatory phenotype during the progression of NASH compared with non-lipid-associated macrophages[13,52], a 2021 study showed that the CCR2-dependent lipid-associated macrophages are anti-fibrogenic and protective, residing in hepatic crown-like structures[61]. The increased infiltration of monocyte-derived cells during NASH progression was induced by the loss of embryo-derived Kupffer cells, confirming the need for an available niche for monocytes to infiltrate the liver. While Kupffer cells during NASH have been characterized into subsets of either *Trem2*^{high}, with a similar phenotype to lipid-associated macrophages, and *Trem2*^{low} as well as embryo-derived and monocyte-derived subsets[13,52,54,57], a 2021 study revealed an embryo-derived Kupffer cell subset, called Kupffer cell 2 (CD206^{high}ESAM^{+}), that has a crucial role in liver oxidative stress associated with obesity via the expression of CD36 (REF.[55]).

Comparison of single-cell transcriptomic profiles of human and mouse liver macrophages reveals that mouse lipid-associated macrophages overlap with the *TREM2*$^{+}$*CD9*$^{+}$ scar-associated macrophages in humans, indicating that these two groups might represent the same cell population[52]. In humans, the proportion of scar-associated macrophages are dramatically increased during liver cirrhosis compared with the steady state[45]. In silico temporal trajectory analysis of single cells further indicated that scar-associated macrophages are derived from circulating monocytes, as previously observed in mice[13,52,57]. Scar-associated macrophages also accumulate in the liver fibrotic niche and upregulate a group of pro-fibrogenic genes including *SPP1*, *LGALS3*, *CCL2*, *CXCL8*, *PDGFB* and *VEGFA*[45]. Overall, single-cell technologies have been crucial in identifying the effect of a NASH-inducing diet on the acquisition of a pro-fibrogenic phenotype by liver macrophages, although additional functional studies and translatability to pathogenesis in humans would be of great interest.

While most of these studies revealed a detrimental role of monocyte-derived macrophages in both mice and humans, a 2021 study suggested that embryo-derived liver macrophages contribute to oxidative stress associated with hepatic steatosis[55]. These differences in contribution of embryo-derived or monocyte-derived macrophages to metabolic disease might be due to different, or even opposing, roles of liver macrophage subsets throughout the progression of NAFLD pathogenesis rather than species differences or variability due to the different mouse models used. Addressing this question would be quite challenging as prospective studies are rarely possible in humans. Analyses of liver macrophages in different cohorts with various degree of NAFLD severity might, however, provide insights into the profile of macrophages during NAFLD progression.

Liver macrophage cellular interactions in NAFLD. Inter-organ and intra-organ crosstalk are particularly important in the context of metabolic diseases in which metabolic tissues could completely modify their secretome profiles. For instance, the severity of hepatic steatosis has been strongly linked to adipose tissue insulin resistance in NAFLD[107], highlighting the importance of crosstalk between adipose tissue and the liver during NAFLD[108,109]. Unfortunately, although the literature suggests that crosstalk between endocrine tissue, metabolic organs and liver macrophages is important for NAFLD and NASH, few studies have addressed the direct role of such crosstalk on liver macrophages, especially during metabolic diseases such as NAFLD. Most studies have mainly focused on metabolic organ-derived factors regulating hepatocyte function. These have been very well described and include, for example, extracellular vesicles, fatty acids, adipokines released from adipose tissue, myokines released from muscle, gut-derived factors and hormones produced by the pancreas[110]. Interestingly, leptin signalling in liver macrophages has been

suggested to be the mechanism mediating the beneficial effect of leptin on liver function including improved lipid metabolism. Myeloid cell-specific deletion of the leptin receptor in mice prevented the acute effects of leptin on liver lipid metabolism, while the metabolic effects of leptin were maintained in mice lacking leptin receptor expression in hepatocytes[111]. Novel strategies to manipulate the expression of genes specific to Kupffer cells[53,55,90,93,112], and the notion that these cells are important for liver function, might expand our knowledge of how inter-organ crosstalk affects the response of liver macrophages to metabolic organs in NAFLD.

Liver macrophage interactions with surrounding cells have been studied at different stages of NAFLD in mice. These studies have revealed inflammatory and non-inflammatory crosstalk, mainly depending on the duration of the diet inducing NAFLD and therefore its severity, with inflammatory crosstalk being more frequent during severe stages of NAFLD. All of these studies have found cellular interactions between liver macrophages and surrounding liver cells or between liver cells and macrophages in both mice and humans[113]. In this Review, we focus our attention on cell-to-cell interactions that have been experimentally validated using systems such as cell-specific gene manipulation or co-culture models in the context of NAFLD (FIG. 4). While surrounding cells mainly regulate the recruitment and differentiation of monocytes, liver macrophages have been shown to be important for the regulation of several cellular functions such as production of extracellular matrix (for example, during fibrosis)[97,100,114–116] and infiltration of natural killer T cells[117]. Although, studies of cell-to-cell communication in human liver are limited, Ramachandran et al. performed ligand–receptor analyses using scRNA-seq to study the interactions of cells during cirrhosis[45]. They found that scar-associated macrophages induce the proliferation of hepatic stellate cells via secretion of TNFSF12 and PDGF-BB, which bind to their cognate receptors on hepatic stellate cells. This study suggested that a distinct population of macrophages could be important for the development of fibrosis in NASH. However, further functional studies inhibiting signalling of these factors using genetic manipulation or receptor antagonists would be necessary to validate the importance of these molecules in interactions between macrophages and other liver cells and the role of such cell-to-cell communication in the progression of NAFLD.

Additional functional studies are clearly required to further understand the cellular and molecular mechanisms involved in the development of liver fibrosis. For example, whether targeting the inflammatory or non-inflammatory functions of liver macrophages or factors expressed by distinct liver macrophage populations during NAFLD progression to NASH represent a promising therapeutic strategy remains to be demonstrated.

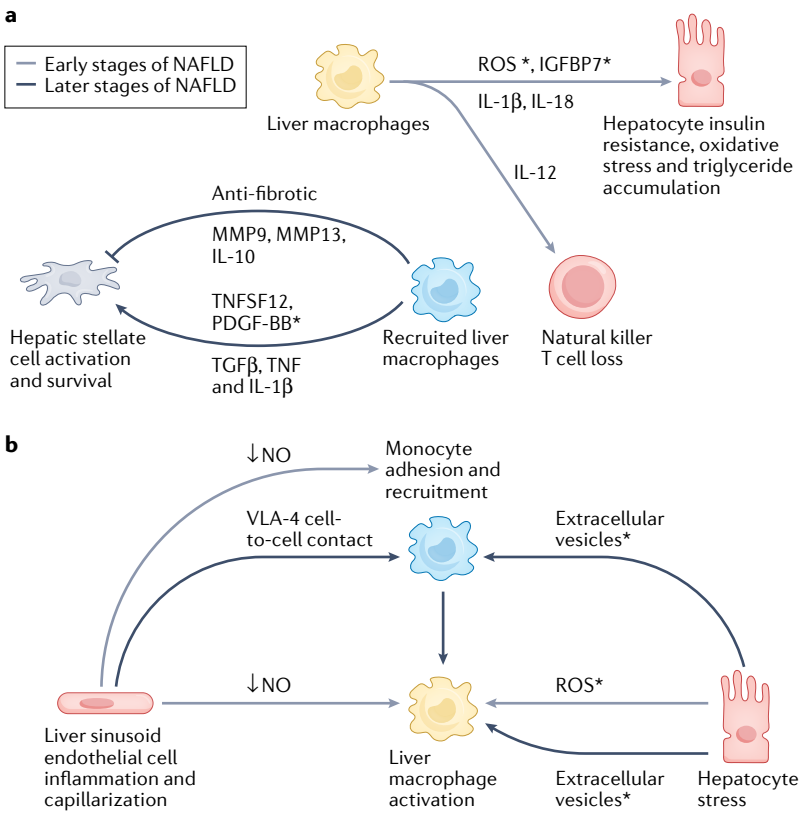


Fig. 4 | **Key cellular interactions between liver macrophages and other liver cell types in NAFLD and NASH. a** | Cell signalling from macrophages to other liver cell types in mice. In early NAFLD, macrophages regulate the loss of natural killer T cells via IL-12 (REF.[117]), insulin signalling[90], oxidative stress[93] and lipid (triglyceride) accumulation[82,118] in hepatocytes via IGFBP7, ROS and IL-1β or IL-18. Recruited macrophages can regulate hepatic stellate cell activation and proliferation via TGFβ[100,114], TNF and IL-1β[115]. These have also been shown to have anti-fibrotic functions by secretion of matrix-degrading metalloproteases[97,116]. **b** | Cell signalling from other liver cell types to macrophages. During early stages of NAFLD, liver sinusoidal endothelial cells induce recruitment of monocyte-derived macrophages and inflammatory activation of macrophages by reduced secretion of NO[119,120]. Hepatocytes can also induce activation of macrophages by ROS[93]. However, during later stages of NAFLD, hepatocytes induce recruitment of monocytes and activation of liver macrophages by secreting inflammatory extracellular vesicles, which are taken up by macrophages[118,121]. At later stages, liver sinusoid endothelial cells induce recruitment of monocytes by upregulating adhesion molecules (such as VLA-4)[122]. The asterisks indicate interactions that are conserved in humans. MMP, matrix metalloproteases; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NO, nitric oxide; ROS, reactive oxygen species.

Conclusions

Studies exploring the cellular diversity of macrophages in the liver have highlighted the changes that occur in their composition, function and turnover during NAFLD progression. In particular, scRNA-seq analyses have provided compelling evidence for the alteration of the transcriptional profiles of distinct liver macrophage subsets during the severe stages of liver disease pathologies, including fibrosis and cirrhosis. However, we do not yet know whether macrophages have an active role in the initiation of disease progression from simple hepatic steatosis to NASH and fibrosis. Moreover, while various clustering methods are available for scRNA-seq analyses, identifying novel cell populations and causative gene regulation requires caution due to the individual variations and noise associated with the use of these techniques

As all of these scRNA-seq studies have used different nomenclature to describe macrophage subsets in the liver, a unified terminology of macrophage populations

during health and NAFLD and/or NASH would aid in comparing different studies. Additional functional studies, ideally with a targeted approach to deplete specific liver macrophage subsets or genetic manipulation of the factors they express, are needed for defining the contributions of macrophage populations to NAFLD pathogenesis. The rapid pace of data generation using multi-omics approaches has had both advantages and disadvantages. A major advantage of this technology has been providing a thorough characterization of macrophage diversity; however, it has often created substantial challenges for clinical translatability. Nevertheless, these unbiased approaches have indicated the multiple roles of liver macrophages outside their inflammatory function in the development of metabolic diseases. Advances in the characterization of macrophage zonation, cellular metabolism and cell-to-cell interactions including inter-organ communication with other metabolic tissues, in parallel with functional validation, will be key to the elucidation of the specific roles of liver macrophage subpopulations during the complete spectrum of NAFLD and NASH pathogeneses.

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